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# Target site mutations and metabolic detoxification of insecticides in continental populations of *Cimex lectularius* and *Cimex hemipterus* (Hemiptera: Cimicidae)

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In recent decades, the common and the tropical bed bugs have experienced a resurgence in many parts of the world. The evolution of insecticide resistance in bed bug populations is considered a significant factor contributing to this resurgence. We analyzed samples of *Cimex lectularius* L. and *Cimex hemipterus* (F.) from Europe (Spain 41, Switzerland 2, the Czech Republic 1), Asia (Hong Kong 34), North America (USA 14, Mexico 3), and South America (Colombia 3) to assess the prevalence and mechanisms of insecticide resistance. We identified specimens morphologically and barcoded them by sequencing the mitochondrial Cytochrome c oxidase subunit I (*COI*) and the *16S* ribosomal RNA (*16S* rRNA) genes. Additionally, we screened segments of the voltage-gated sodium channel (*VGSC*) and the nicotinic acetylcholine receptor (*nAChR*) genes for point mutations associated with insecticide resistance and measured the activity of detoxifying enzymes. All samples from North America and Europe were identified as *C. lectularius*, whereas specimens from Hong Kong were *C. hemipterus.* Out of 64 *C. lectularius* samples tested for knockdown resistance (*kdr*) mutations, 90.6% contained at least 1 known mutation. All 35 *C. hemipterus* samples exhibited *kdr* mutations. A new mutation was identified in the pyrethroid target site in both common (F1524C) and tropical (F1450C) bed bugs. No resistance-associated mutations in the *nAChR* gene were found. Several populations that exhibited *kdr* mutations also showed elevated activity of detoxifying enzymes. The high frequency of *kdr*-associated mutations in bed bug populations from Spain and Hong Kong limits the efficacy of pyrethroids for their control.

*Key words:* bed bug, urban pest, insecticide resistance, knockdown resistance, metabolic resistance

## Introduction

Over the last 2 decades, infestations of both *Cimex lectularius* L. and *Cimex hemipterus* (F.) (Hemiptera: Cimicidae) have increased dramatically in numerous parts of the world and have become a challenging indoor pest to control (Doggett et al. 2004, Potter 2006). Historically, *C. lectularius* has been reported in the temperate regions, whereas *C. hemipterus* has been observed in the tropical and subtropical regions, with the Tropics of Capricorn and Cancer serving as approximate latitudinal limits (Usinger 1966). Both species are found in Australia (Doggett and Cains 2018), Thailand (Tawatsin et al. 2011), China (Wang et al. 2013), and tropical and subtropical regions of the United States (Campbell et al. 2016, Lewis

et al. 2020). Recently, changes in the geographical distribution of *C. hemipterus* have been reported in some European countries (Balvín et al. 2021).

The resurgence of bed bugs has been primarily associated with increased international travel and commerce as well as the evolution of insecticide resistance due to strong selection pressures exerted by the continuous use of pyrethroid-based treatments for infestation control (Romero et al. 2007, 2018, Lewis et al. 2023). Pyrethroid resistance in *C. lectularius* has been reported at high frequencies for bed bug populations in Europe, America, and Asia (Dang et al. 2015a, Romero 2018). Similarly, *C. lectularius*, insecticide resistance in *C. hemipterus* has been reported in several countries, including Tanzania (Myamba et al. 2002), Sri Lanka (Karunaratne et al. 2007, Punchiwewa et al. 2019), Australia, India, Kenya, Malaysia, and Thailand (Dang et al. 2015b), suggesting that resistance might be widespread.

Extensive evidence exists for the existence of multiple mechanisms of the insecticide resistance in *C. lectularius*. These mechanisms include knockdown resistance (*kdr*)-associated mutations in the voltage-gated sodium channel (*VGSC*), which reduce the affinity of the insecticide to the target site (target-site insensitivity); increased metabolic detoxification by cytochrome P450 monooxygenases (P450s), esterases, and Glutathione-S-Transferases (GSTs); and reduced cuticular penetration of the insecticides (Zhu et al. 2013, Dang et al. 2017, Romero 2018). In *C. lectularius*, 3 mutations in the *VGSC* gene (V419L, L925I, and I936F) have been identified (Yoon et al. 2008, Dang et al. 2015a) and their frequency and distribution have been extensively studied globally (Zhu et al. 2010, Booth et al. 2015, Palenchar et al. 2015, Dang et al. 2017, Balvín and Booth 2018, Holleman et al. 2019, Akhoundi et al. 2021, Lewis et al. 2023). Four *kdr* mutation haplotypes have been described for *C. lectularius*: haplotype A (no V419L or L925I mutations), haplotype B (only L925I), haplotype C (both V419L and L925I mutations), and haplotype D (only V419L). If the I936F mutation is present, some researchers add a superscript, for example, $A^b$ (I936F) or $B^b$ (L925I and I936F) (Holleman et al. 2019).

A recent study showed that over the past 10 years, there has been an increase in the frequency of bed bug populations exhibiting multiple *kdr*-associated mutations, and this has been related to the continued use of pyrethroids (Lewis et al. 2023). Similar resistance mechanisms to pyrethroids have been reported in *C. hemipterus* (Dang et al. 2017), including 11 *kdr*-associated mutations: L1014F (Dang et al. 2015b, Punchihewa et al. 2019, Ghavami et al. 2021, Soh and Singham 2021); M918V (Dang et al. 2015b, Ghavami et al. 2021, Soh and Singham 2021); D953G (Dang et al. 2015b, Ghavami et al. 2021); L899V (Dang et al. 2015b); Y/I995H, V1010L, I1011F, V1016E, and L1017F/S (Punchihewa et al. 2019); and I1011T and A468T (Ghavami et al. 2021). Notably, the frequency and distribution of *kdr* mutations in *C. hemipterus* are less investigated (Dang et al. 2017), even in highly populated countries such as China, where the prevalence of this species is high (Wang et al. 2015).

In the last decade, neonicotinoids combined with pyrethroids have been used extensively for bed bug control in the United States and other parts of the world (Lee et al. 2018). Shortly after the introduction of these combinations, some bed bug populations in the United States were reported to be resistant to neonicotinoids. Romero and Anderson (2016) demonstrated high activity of detoxification enzymes in field-collected bed bug populations that exhibited high resistance ratios in bioassays with various neonicotinoids. Target-site mutations in nicotinic acetylcholine receptor (*nAChR*) subunits represent another important neonicotinoid resistance mechanism reported in other insects (Xu et al. 2022a). However, to our knowledge, there are no reports of mutations in the *nAChR* gene in bed bugs.

Expanding studies on the identification of bed bug species and assessing their insecticide resistance status are crucial for formulating effective management strategies aimed at curbing their local and global spread. In this study, we collected bed bugs in the United States, Mexico, Colombia, Hong Kong, Spain, Switzerland, and the Czech Republic and conducted morphological and molecular identification of samples as well as barcoding of the *VGSC* and *nAChR* genes. Characterization of the activity of detoxifying enzymes was also conducted in bed bugs collected from the United States, Mexico, and the Czech Republic.

## Materials and Methods

### Sample Collection

Collections were made by local pest management services from different urban settings or provided by US laboratories (see Acknowledgments). Samples from Mexico, United States, and the Czech Republic were obtained from laboratory colonies (New Mexico State University, North Carolina State University, Rose Pest Solutions), which were established from specimens collected between 2008 and 2022. All samples from Spain, Hong Kong, Switzerland, and Colombia were collected in 2022; 95% of the samples from Spain were collected in the autonomous community of Madrid, from multi-housing units and several rooms within social shelters (Fig. 1A, Supplementary Material 1). In Hong Kong, all bugs were collected from family flats in high-rise buildings located in different parts of the city (Fig. 1B, Supplementary Material 1). Specimens were preserved in 70% ethanol and at – 80°C until their use. For control, bed bugs from the Fort-Dix strain were used. This bed bug strain has not been exposed to insecticides for ~30 yr.

### Species Identification

Morphologically, samples were identified to the species level by observing morphological characters of the pronotum with a stereomicroscope (Olympus SZ61, Olympus Corporation, Tokyo, Japan). *Cimex lectularius* has a domed pronotum with a lateral and wider margin, when compared with that of *C. hemipterus* (Usinger 1966, Golub et al. 2020) (Fig. 2).

For DNA barcoding, Genomic DNA was extracted from a single specimen per collection site. DNA extraction of adult bed bug specimens was performed using DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA) with modifications in the proteinase K time (4 h). All samples were subjected to molecular analysis to confirm identification by sequencing a 428-bp fragment of mitochondrial *16S* rRNA gene (Simon et al. 1994, Kambhampati and Smith 1995) and a 650-bp fragment of the cytochrome c oxidase subunit 1 (*COI*) gene (Booth et al. 2015) (Table 1).

Polymerase chain reaction (PCR) conditions for *COI* primers were as follows: initial denaturation for 2 min at 95°C; 35 cycles of 40 s at 94°C, 40 s at 42°C, and 1 min at 72°C; and extension for 5 min at 72°C. PCR conditions for *16S* rRNA were as follows: initial denaturation for 2 min at 95°C; 35 cycles of 45 s at 94°C, 45 s at 46°C, and 1 min at 72°C; and extension for 5 min at 72°C. Nucleotide sequence editing and analysis with amino acid sequence prediction were performed using MEGA11 software (DNAStar, Madison, WI, USA). The consensus sequences were compared with the sequences publicly available from GenBank using the National Center for Biotechnology Information (NCBI) nucleotide BLAST function to check identity.

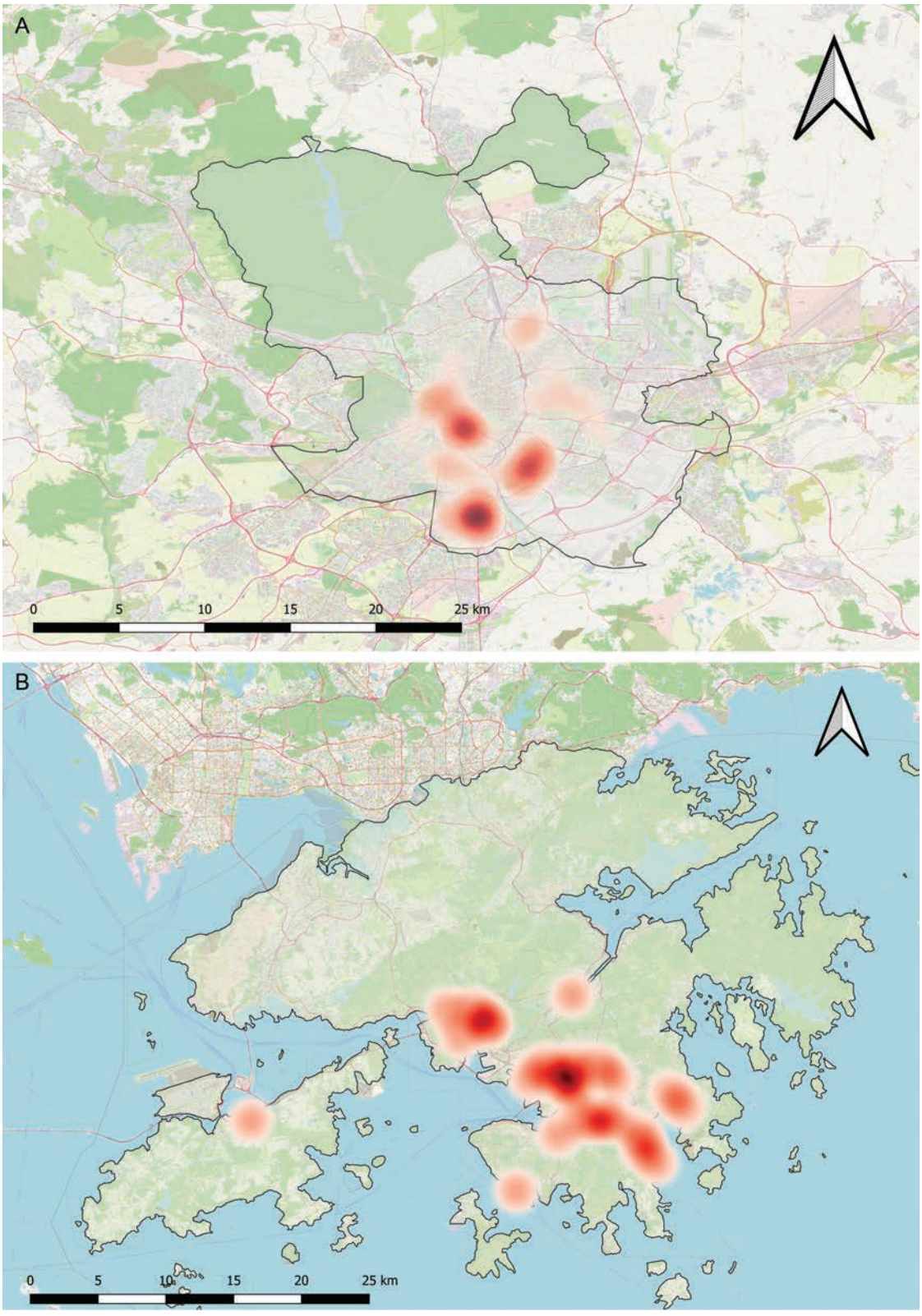


**Fig. 1.** Heat maps demonstrating the geographical location where bed bugs were collected in Madrid, Spain (A) and Hong Kong (B).

## Sequence Analysis of *VGSC* and *nAChR* Subunit Genes

One specimen of *C. lectularius* or *C. hemipterus* from each collection was screened for the presence of *kdr*-related mutations and mutations in *nAChR* subunit genes. For the screening of *kdr* mutations, 3 regions of the *VGSC* genes were amplified (GenBank accession number XM_024226724): the first region (the domain IS6 and the partial domain I–II interlinker) contains mutation sites

(V419L in *C. lectularius* and A468T in *C. hemipterus*) and was amplified with the primer combination FKDR1/RKDR1 (Ghavami et al. 2021) (Table 2); the second region (domain II and the partial domain II–III linker) contains the L925I mutation and I936F putative mutation in *C. lectularius* and L899V, M918I, D953G, Y/L995H, V1010L, I1011F, L1014F, A1007S, I1011T, V1016E, and L1017F/S in *C. hemipterus* and was amplified with the primer combination FKDR2/RKDR2 (Ghavami et al. 2021) (Table 2); and the third region corresponds to the III–IV interlinker region in which the mutation F1534C has been reported in other arthropods, specifically in mosquitoes (Modak and Saha 2022) and was amplified for both bed bug species with the primer pair FKDR3/RKDR3 (Table 2).

**Fig. 2.** Morphological differences between *C. lectularius* (male, left) and *C. hemipterus* (male, right). Arrows indicate the margin of the pronotum, which is wider in *C. lectularius*.

Regions of the *nAChR* subunits α and β were amplified with gene-specific primers generated from the predicted gene sequences of *C. lectularius*. For the α amplified fragment, the predicted length was of approximately 1,604 nucleotides and 534 amino acids (total nucleotide length of α region was 2,767) (GenBank accession number for α XM_014389050.2). For the β amplified fragment, the predicted length was of approximately 1,511 nucleotides and 503 amino acids (total nucleotide length of β region was 2,546) (GenBank accession number XM_014400945.2).

Three sets of primers were designed and used: 1 to analyze the B-loop of the α subunit and 2 to analyze the D-loop of the β subunit (Table 2). For the α subunit, the B-loop was amplified because it contains the Y151S mutation reported in the brown planthopper *Nilaparvata lugens* Stål (Hemiptera: Delphacidae), a mutation that confers resistance to the neonicotinoid imidacloprid (Liu et al. 2005). For the β subunit, different sets of primers were used in each bed bug species (Table 2). This β subunit region was amplified because it contains the R81T and V101I mutations reported in the green peach aphid *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), which have been associated with neonicotinoid resistance (Xu et al. 2022b). In *C. hemipterus*, for the *nAChR* genes, sequences were compared with each other and with susceptible *C. lectularius* because there are no published sequences of these subunits for *C. hemipterus*.

All reactions were carried out with a final volume of 25 µl with the following mixture: 12.5 µl of Taq 2X Master Mix, 0.5 µl of each primer (0.1 µM) (upstream, downstream), and 2 µl of genomic DNA (10–30 ng) (Chebbah et al. 2021). PCR products were

**Table 1.** Oligonucleotide primer sets for the PCR amplification of the mitochondrial *16S* rRNA gene and *COI* gene of *C. lectularius and C. hemipterus*

| Gen | Original name | Sequence | **Fragment size (bp)** | Temperature (°C) | Reference |
|---|---|---|---|---|---|
| *mtDNA rRNA 16S* | LR-J-13007 | 5′-TTACGCTGTTATCCCTAA-3′ | 428 | 46 | Simon et al. (1994), Kambhampati and Smith (1995). |
| | LR-N-13398 | 5′-CGCCTGTTTATCAAAAACAT-3′ | | | |
| *mtDNA COI* | LepF | 5′-ATTCAACCAATCATAAAGATATNGG-3′ | 650 | 42 | Booth et al. (2015). |
| | LepR | 5-TAWACTTCWGGRTGTCCRAARAATCA-3 | | | |

**Table 2.** Oligonucleotide primer sets for the PCR amplification of cDNA fragments of *VGSC* and *nAChR* genes of *C. lectularius and C. hemipterus*

| Primer | Sequence | Fragment size (bp) | **Temperature** (°C) | Reference |
|---|---|---|---|---|
| KDR1 (I-II IL) | 5′-GTCCGTGGCACATGTTGTTCTTCA-3′<br>5′-CTGATGGAGATTTTGCCACTGATGC-3′ | 291 | 55 | Zhu et al. 2010, Ghavami et al. 2021 |
| KDR2 (II-III IL) | 5′-GGAATTGAAGCTGCCATGAAGTTG-3′<br>5′-TGCCTATTCTGTTCGAAAGCCTCAG-3′ | 622 | 55 | Zhu et al. 2010, Ghavami et al. 2021 |
| KDR3 (III-IV IL) | 5′-CGTCCCCTTAGGGCAATGTC-3′<br>5′-CCGTGGTCGTGGAATAGCTT-3′ | 603 | 55 | |
| ALFA | 5-ACGTCGCAATATCCATCGCT-3<br>5-GATAGGGCTCTTCGCAGCAA-3 | 533 | 55 | |
| BETA1 | 5′-CACGCAAAGATGGCTCAATTAC-3′<br>5′-CATCACTTCGCAGATCACATAGA-3′ | 729 | 55 | |
| BETA2 | 5′-AAGGTGCATCACTTCGCAGA-3′<br>5′-AGGAACTTCTCATGGATTTTTCAG-3′ | 358 | 55 | |
| CH BETA1[a] | 5′-AGCAATATGCTCCGAGGAC-3′<br>5′-ACCAAAATGATCTGTAACGACC-3′ | 350 | 55 | |
| CH BETA2[a] | 5′-TGTATTCAGGAGTGATTTGCC-3′<br>5′-TCTGGTTTCCACACTTTGTC-3′ | 180 | 53 | |

[a]Primers used for *C. hemipterus* only.

visualized on 2.5% agarose gel stained with SYBR Green (Thermo Fisher Scientific, Waltham, MA, USA) and UV light transilluminator. PCR conditions were as follows: initial denaturation for 2 min at 95°C; 35 cycles of 40 s at 94°C, 40 s at 55°C, and 1 min at 72°C; and extension for 5 min at 72°C. The melting temperature (*Tm*) was changed depending on the primer pair. Primer pairs, *Tm* and expected amplicon size are provided in Table 2.

## DNA Purification and Sequencing

PCR products were purified using the PCR Purification kit Qiaquick (Qiagen). The primers and temperature used for amplification were the same as those used for sequencing. Sequence review was done in BioEdit 7.7. Nucleotide sequence editing and analysis with amino acid sequence prediction were performed using MEGA11 software (DNAStar). Presence or absence of mutations was scored by eye and sequence alignment. Afterward, the prevalence and types of mutations were recorded.

## Phylogenetic Tree Construction

The phylogenetic tree was constructed by using the neighbor-joining method (Saitou and Nei, 1987) with MEGA11 software (Pennsylvania State University, University Park, PA, USA) from *COI* and *16S* concatenated consensus sequences of the common and tropical bed bug specimens collected in the present study, using *Rhodnius prolixus* Stål (Hemiptera: Reduviidae, accession number AF449138) and *Lygus elisus* Van Duzee (Heteroptera: Miridae, accession number MG944328) as the outgroup. Branches corresponding to partitions replicated in less than 50% of bootstrap replicates (10,000 replicates) were collapsed (Felsenstein 1985). Evolutionary distances were calculated using the p-distance method (Tamura et al. 2004). The tree substitution method was per-nucleotide using the Tamura et al. (2004) method coupled with a heuristic nearest-neighbor-interchange process. The neighbor-joining method was chosen because of its rapidity and the fact that it has a good accuracy that is not affected by a large number of sequences (Tamura et al. 2004). Furthermore, the neighbor-joining method produced similar results to those achieved by a maximum-likelihood methodology (data not shown), and it has been used to evaluate bed bug populations (including endosymbionts) in recent studies (Chebbah et al. 2023). The accession numbers of the different Cimicidae species and the outgroup species were as follows—*Aphrania elongata* Usinger: *COI* MG596812, *16S* rRNA MG596849; *Cacodminae* sp. OB 2013 Makhado: *COI* KF018764, *16S* rRNA KF018730; *Cacodmus vicinus* Horváth: *COI* KF018762, *16S* rRNA KF018728; *Cimex adjunctus* Barber: *COI* KR035747.1, KR032514.1, GU985537, *16S* rRNA GU985557.1, GU985558.1, GU985559.1; *Cimex antennatus* Usinger & Ueshima: *COI* KF018760, *16S* rRNA KF018732.1; *Cimex hemipterus* (F.): *COI* MN915234.1, MN915225.1, MN915224.1, *16S rRNA* MT362654.1, MT362653.1, MT362651.1; *Cimex japonicus* Usinger: *COI* KC503541.1, *16S* rRNA KF018727.1; *Cimex latipennis* Usinger & Ueshima: *COI* KF018758.1, KF018757.1, *16S* rRNA KF018733.1, KF018758.1; *Cimex lectularius* L.: *COI* MN271345.1, MF161526.1, *16S* rRNA MN265385.1, KF018726.1; *Cimex pilosellus* Horváth: *COI* KF018759.1, *16S* rRNA KF018731.1; *Cimex pipistrelli* Jenyns: *COI* KC503529.1, GU985528.1, MK140095.1, MK140092.1, KC503517.1, GU985530.1, MF161528.1, KC503539.1, *16S rRNA* MG596872.1, KF018727.1, GU985552.1, GU985564.1, MG596871.1, GU985549.1, JQ782770.1; *Cimex* sp. OB2010: *COI* GU985542.1, *16S rRNA* GU985564.1; *Lygus elisus* Van Duzee: *COI* MG944328.1, *16S* rRNA AY252785.1; *Oeciacus hirundinis* Lamarck: *COI* MG596809.1, GU985543.1, *16S* rRNA MG596846.1, GU985565.1; *Oeciacus vicarious* Horváth: *COI* KF018753.1, GU985541.1, KF018753.1, *16S* rRNA KF018722.1, GU985563.1; *Paracimex setosus* Ferris & Usinger: *COI* KF018761.1, *16S* rRNA KF018735.1; and *Rhodnius prolixus* Stål: *COI* AF449138.1, *16S* rRNA AF324519.1.

## Detoxification Enzymes Assays

### Protein Preparations

Three starved adult male bed bugs were homogenized in 1 ml of cold 0.1 M sodium phosphate buffer (pH 7.0), following a methodology outlined in Gaire et al. (2020), with 3 replicates per collection. The insect homogenates were then centrifuged at 10,000 × g for 20 min at 4°C in an Eppendorf 5430 R Refrigerated Centrifuge (Enfield, CT, USA). The resulting supernatants, serving as enzyme sources, were used to assess detoxification enzyme activity. Protein concentrations were determined using Bradford (1976) colorimetric assays with bovine serum albumin as a standard. Concentrations were measured at 595 nm using an Agilent BioTek Synergy HTX Multi-Mode Microplate Reader spectrophotometer. The samples were stored at – 80°C until further use. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### P450 Assay

P450 activity was evaluated utilizing p-nitroanisole (p-NA) as the substrate (Rose et al. 1995, Liu et al. 2015). The reaction mixture consisted of 50 µl of p-NA (0.3 M in acetonitrile) in 10 ml of sodium phosphate buffer at pH 7.0, conducted in transparent 96-well microplates. Each treatment well received 10 µl of protein extract and 10 µl of fresh NADPH solution, whereas control wells were given an equivalent volume of sodium phosphate buffer. Reactions were initiated by adding 200 µl of sodium phosphate buffer containing 1 mM p-NA to all wells. Monitoring reactions at 405 nm for 5 min was done using a SpectraMax2 spectrophotometer (Molecular Devices, San Jose, CA, USA). Specific activity was calculated using the extinction coefficient for the resulting product p-nitrophenol (6.53/mM/cm) and expressed as nmol/min/mg protein.

### Esterase Activity

The methodology for this assay followed that used by Gaire et al. (2020). The activity of this enzyme was evaluated using p-nitrophenyl acetate as a substrate (Wu et al. 1998, Gaire et al. 2020). Monitoring reactions at 405 nm for 5 min was done using an Epoch 2 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Specific activity was calculated using the extinction coefficient for the end product p-nitrophenol (6.53/mM/cm) and expressed as nmol/min/mg protein (Wu et al. 1998).

### GST Activity

The specific assay methodology used for this enzyme followed that used by Gaire et al. (2020), where chloronitrobenzene was used as a substrate (Wu et al. 1998, Gaire et al. 2020). Reactions were monitored for 5 min at 344 nm using an Epoch 2 spectrophotometer (Agilent Technologies). The extinction coefficient of 9.5/mM/cm for the end product S-(2,4-dinitrophenyl) glutathione was utilized to compute specific activity (nmol/min/mg protein) (Wu et al. 1998).

## Statistical Analysis

Enzymatic activity data were subjected to analysis of variance once the normality of the data was verified. Dunnett's test was used to compare enzymatic activity between samples and the control (the susceptible Fort-Dix strain that was used as reference/baseline). *P*-value

corrections were performed using the methods of Holm (1979) and Benjamini and Yekutieli (2001). All analyses and visualizations were performed with the statistical package R (Version 4.2.3, Shortstop Beagle) in the RStudio environment, version 1.2.5 (Boston, MA, USA).

## Results

### Morphological Identification

From the 99 samples morphologically examined, 64 were identified as *C. lectularius*, whereas 35 were *C. hemipterus* (Fig. 2, Table 3). All adult specimens collected in the Czech Republic, Mexico, Spain, Switzerland, and the United States were identified as *C. lectularius*. In contrast, all samples collected in Hong Kong were identified as *C. hemipterus*. Both *C. lectularius* (3 samples) and C. *hemipterus* (1 sample) were found in Colombia (Table 3).

### DNA Barcoding

Molecular identification was confirmed for all specimens through the sequencing of a 430-bp fragment of the *16S* rRNA gene and a 650-bp fragment of the *COI* gene. These samples typically had a 98%–100% identity with the top results of other samples stored in GenBank for *C. lectularius* (Supplementary Material 2). For *C. hemipterus*, identity was higher when the sequences were compared with those top sequences stored in GenBank, with 99%–100% identity. Most nucleotide differences ranged from 1 to 10 nucleotides. In both species, molecular analysis matched 100% phenotype-based identification.

The phylogenetic analysis, utilizing concatenated *16S* rRNA and *COI* gene sequences, categorized the *Cimex* samples into 2 distinct branches: *C. lectularius* and *C. hemipterus* (Fig. 3). Within the *C. lectularius* branch, 2 primary clusters were identified, with both clusters containing a similar proportion of *C. lectularius* samples collected from Spain. The majority of US samples were included in the first cluster, with 2 out of 3 Mexican samples also grouped in this cluster. The second cluster comprised samples from Spain (15), the United States (3), and Colombia (3). Within the *C. hemipterus* branch, 2 distinct clusters were identified: 1 included the Hong Kong samples closely related to previously sequenced Chinese samples, and the other included all but 1 sample from Colombia (Fig. 3).

### Identification of Mutations in the *VGSC* in *C. lectularius*

Samples of *C. lectularius* collected in Europe (Spain, the Czech Republic, and Switzerland) and the Americas exhibited a high frequency of *kdr*-associated mutations (Table 4, Supplementary Material 3). From 41 *C. lectularius* samples collected in Spain, 87.8% had at least 1 *kdr*-associated mutation, 4.8% of which possessed the I936 mutation (haplotype $A^b$), 73.1% the L925 (Haplotype B), and only 9.7% had both L925 and V419L mutations (haplotype C) (Table 4). Five Spanish samples (12.1%) did not exhibit the *kdr*-associated mutations investigated. Samples collected in the Czech Republic (1) and Switzerland (2) were all haplotype B (Table 4). American samples were mostly represented from specimens collected in the United States (14), and 92.8% of these samples had *kdr*-associated mutations. In total, 1 sample (7.1%) (collected in Cincinnati) did not have *kdr* mutations, 1 had L925 mutation (7.1%), whereas the remaining 12 (85.7%) possessed both L925 and V419 mutations (Table 4). Among the American samples, 3 were collected from poultry farms (Concord, Statesville, and Winfield) all of which were haplotype C (Table 4, Supplementary Material 3). One sample from Mexico had haplotype B, whereas the other 2 had haplotype C. In turn, 1 Colombian sample exhibited the haplotype $A^b$, 1 haplotype haplotype $B^b$, and 1 haplotype C (Table 4, Supplementary Material 3). In total, 5 samples of *C. lectularius* collected in Madrid Spain (B3.21, B2.13A, B1.2, B1.38), and 1 from Chiquinquirá, Colombia, exhibited an additional homozygous mutation at position 1524 in the III–IV Interlinker region of *VGSC* gene, resulting in a change from phenylalanine to cysteine (F1524C) (Fig. 4A, Supplementary Material 3). For example, sample B1.38 exhibited a mutation at position 1524, changing phenylalanine to cysteine compared with the wild-type *C. lectularius* sequence from GenBank (Fig. 5A). Similar substitution from phenylalanine to cysteine was identified in 9 samples of *C. hemipterus* at position 1450 (F1450C) (Fig. 4B) in the III–IV interlinker region. This mutation was detected in the Hong Kong (HK5, HK6, HK11, HK13, HK14, HK15, HK19, HK23, and HK25) and Medellín sample (MED) (Supplementary Material 3). In the particular case of the *C. hemipterus* MED sample, the mutation at position 1450 was also detected in the wild-type *C. lectularius* (Fig. 5C), *Blatella germanica* (Linnaeus) (Fig. 5D), *Pediculus humanus corporis* (Fig. 5E), *Aedes albopictus* S. (Fig. 5F), and *Aedes aegypti* L. (Fig. 5G). The F1524C mutation was detected in 2 samples with haplotypes $A^+$ (B2.13A and Chiquinquirá), 2 with haplotype B (B1.2 and B1.38), and 1 with haplotype C (B3.21B) (Supplementary Material 3).

The *C. lectularius* samples were mostly homozygous. Four presented heterozygosity for the V419L mutation: 2 from Madrid, Spain (B3.46 and B3.21B), 1 from the United States (Manda), and 1 from Colombia (Bogotá). Four samples presented heterozygosity for the L925I mutation: 3 from Spain (B1.9A, B1.9B, and B3.27), and 1 from the United States (Shanda). No other samples presented heterozygosity in either of those mutations or the I936F and F1524C mutations.

**Table 3.** Species identification of bed bug samples by country using morphological characteristics

| Country | *Cimex lectularius* | *Cimex hemipterus* |
|---|---|---|
| The Czech Republic | 1 | - |
| Colombia | 3 | 1 |
| Hong Kong | - | 34 |
| Mexico | 3 | - |
| Spain | 41 | - |
| Switzerland | 2 | - |
| USA | 14 | - |
| Total | 64 | 35 |

### Identification of Mutations in the *VGSC* in *C. hemipterus*

All *C. hemipterus* samples collected in Hong Kong (34) and Colombia (1) had resistance-associated mutations: M918I, D953G, and L1014F. Four synonymous mutations were detected in 100% of the samples in positions L925, K949, N950, and L436 (chromatograms not shown). All samples presented with homozygosity in the aforementioned mutations. No other resistance-associated mutations previously reported in the *VGSC* in *C. hemipterus* were detected.

### Screening of Nicotinic Acetylcholine Receptors

No resistance-associated mutations were found in the α and β subunits of the *nAChR* gene in either bed bug species. In the α subunit, a synonymous mutation (E151) was found in samples from the

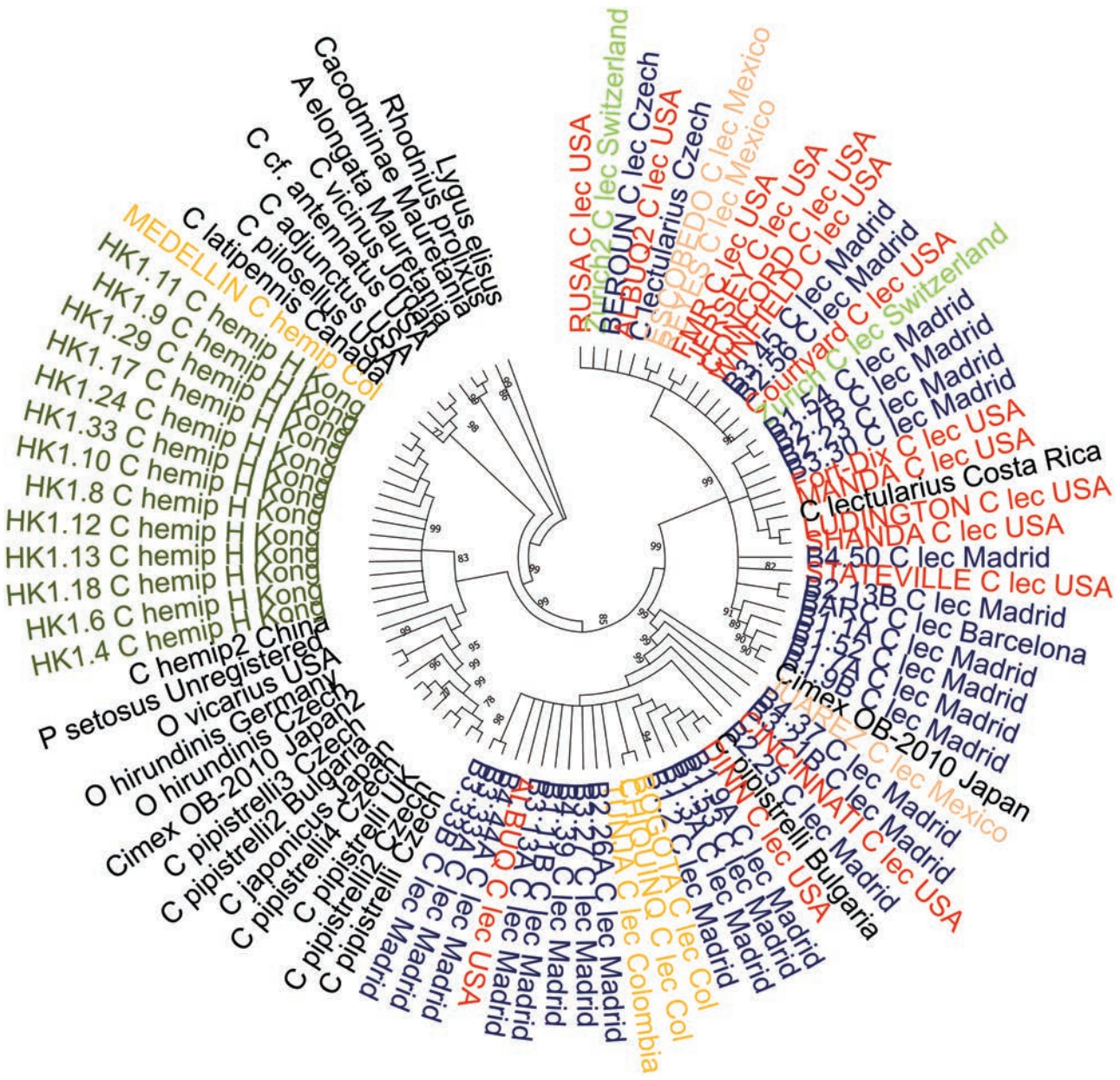


**Fig. 3.** Neighbor-joining (NJ) tree constructed from *COI* and *16S* rRNA concatenated consensus sequences of tropical and common bed bug specimens collected in the present study and from sequences collected from GenBank. Branches represent genetic sequences obtained from GenBank depicting other countries (Costa Rica) or other species.

United States (Albuquerque, Escobedo, Reyes, and Concord), Spain (B1.3A and BARC), and Colombia (Bogotá). Three samples were heterozygous (2 from the United States, Albuquerque and Concord, and 1 from Spain, BARC), and the rest were homozygous (1 from Colombia, 1 from Spain, and the other 2 from the United States) (Fig. 4C). Compared with *C. lectularius*, the *nAChR* α subunit of *C. hemipterus* analyzed with the primer CHβ1 exhibited synonymous mutations at positions G191 and V211 (Fig. 4D), whereas the *nAChR* β subunit exhibited a synonymous mutation at position L59 (Fig. 4E). In the region analyzed with the primer β2, 3 more synonymous mutations were found (G92, G95, and P101). The D-loop region sequence and chromatogram can be seen in Fig. 4E.

### Characterization of Detoxification Enzyme Activities

The cytochrome P450 enzymatic activity in the Cincinnati and Shanda samples was significantly higher (~2.33- and 3.8-fold, respectively) compared with the susceptible Fort-Dix strain ($P < 0.0001$) (Fig. 6). The esterase activity was significantly higher in Escobedo (~4.73-fold), Jersey City (~2.9-fold), Juarez (3.9-fold), Rusa (~2.75), and Statesville (2.75-fold) than in Fort-Dix ($P < 0.001$) (Fig. 6). At last, GST activity was also significantly higher (~4.7-fold) in Escobedo and FMR (~3.14-fold) relative to the Fort-Dix strain ($P < 0.0001$) (Fig. 6).

## Discussion

Bed bug samples collected in all 8 countries were morphologically identified as *C. lectularius* or *C. hemipterus*, and species identifications were confirmed by molecular methods. Our study represents the first investigation into the characterization of the insecticide mechanisms in bed bug populations from Spain, Hong Kong, Mexico, and Colombia. All Spanish samples were identified

**Table 4.** Frequency of *kdr*-associated genotypes reported in *C. lectularius* and resistance associated mutations in *C. lectularius and C. hemipterus* populations sampled in countries of Europe, Asia, and the Americas

| *Cimex lectularius* | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Total | Wild Type (A) | I936F ($A^b$) | L925I (B) | L925I and I936F ($B^b$) | L925I and V419L (C) | V419L (D) |
| Europe | | | | | | | |
| Spain | 41 | 5 (12.1%) | 2 (4.8%) | 30 (73.1%) | 0 (0%) | 4 (9.7%) | 0 (0%) |
| The Czech Republic | 1 | 0 (0%) | 0 (0%) | 1 (100%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Switzerland | 2 | 0 (0%) | 0 (0%) | 2 (100%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Americas | | | | | | | |
| United States | 14 | 1 (7.1%) | 0 (0%) | 1 (7.1%) | 0 (0%) | 12 (85.7%%) | 0 (0%) |
| Mexico | 3 | 0 (0%) | 0 (0%) | 1 (33.3%) | 0 (0%) | 2 (67.7%) | 0 (0%) |
| Colombia | 3 | 0 (0%) | 1 (33.3%) | 0 (0%) | 1 (33.3%) | 1 (33.3%) | 0 (0%) |

| Cimex hemipterus | | | | |
|---|---|---|---|---|
| | Total | Wild Type | M918I, D953G, L1014F | A468T, L899V, Y/L995H, I1011T, V1016E, L1017F/S |
| Americas | | | | |
| Colombia | 1 | 0 (0%) | 1 (100%) | 0 (0%) |
| Asia | | | | |
| Hong Kong | 34 | 0 (0%) | 34 (100%) | 0 (0%) |

as *C. lectularius*. The majority of these collections were made in low-income or transient housing in Madrid, the most populated city in Spain (6.5 million people) with an intense influx of visitors and immigrants in the last 2 decades (Gil-Alonso and Thiers-Quintana 2019). Similarly, China has witnessed a dramatic resurgence of bed bugs in recent decades (Wang et al. 2015, Zhang et al. 2021). Almost all Chinese provinces have reported bed bugs, with the Guangdong Province, the southernmost of mainland China, being the region with the highest proportion of bed bug reports (Wang et al. 2013). In our study, all specimens collected from subdivided flat units in the Hong Kong were morphologically and molecularly identified as *C. hemipterus*. The high mobility of people in urbanized areas is a risk factor associated with the spread of bed bugs (Wilson 2018). Similarly, crowded conditions in transient housing along with financial and organizational constraints make bed bug control challenging in these environments (Schneider 2019).

For the American continent, we analyzed samples from Colombia, Mexico, and the United States. Specimens collected in Colombia were identified as *C. lectularius*. We also identified in Medellín, Colombia, a sample of *C. hemipterus* that confirms the presence of this species in Colombia initially reported in the 1950s (Figueroa 1953). Nevertheless, *C. lectularius* was reported in Colombia nearly a decade ago (Posso et al. 2011).

We constructed a phylogenetic tree using sequences from *Cimex* samples collected from the temperate and tropical countries to gain insights into the evolutionary relationships and assess the genetic divergence across populations. The resulting phylogenetic tree revealed 2 distinct branches, each representing separate genetic lineages corresponding to the 2 *Cimex* species. Within *C. lectularius*, there was higher genetic diversity compared with *C. hemipterus*. This increased diversity within the *C. lectularius* cluster confirms broader genetic variation in mitochondrial DNA within this species (Rosenfeld et al. 2016, Booth et al. 2018), probably influenced by widespread human-mediated dispersal across different regions (Booth et al. 2018). The lack of clear geographical structuring within the *C. lectularius* cluster could be due to the interspersion of samples from various global locations and the limited number of samples analyzed from some countries (Booth et al. 2018, Booth, 2024). In contrast, the *C. hemipterus* cluster, consisting mostly of samples collected in Hong Kong, showed lower genetic diversity and contained 2 small, ill-defined subclusters. One subcluster exhibited a closer genetic relationship to previously documented Chinese samples, whereas the other subcluster showed genetic similarity to samples from Colombia. This unexpected genetic affinity across distant regions could suggest passive transport of *C. hemipterus*, possibly via human activities. However, the relative lack of genetic diversity in *C. hemipterus* for the analyzed genes limits the scope of these results, making it challenging to draw definitive conclusions about their dispersal patterns. These findings underscore the importance of expanding our repertoire of genetic markers to study the bed bug populations (Booth 2024).

The majority of *kdr*-genetic profiles of *C. lectularius* collected from Spain and the United States showed at least 1 of the 2 *kdr*-associated mutations, V419L and L925I. Similar genetic profiles were identified in the European and Latin American countries. However, the limited number of samples screened prevents us from drawing conclusions about the current status of these mutations in these countries. Bed bug populations possessing V419L and L925I mutations are associated with high levels of pyrethroid resistance (Yoon et al. 2008, Zhu et al. 2010). A third mutation, I936F, that confers low-to-medium levels of resistance to pyrethroids has been detected in bed bug populations from Australia, Israel, and Europe, and recently in the US city of Albuquerque, NM (Dang et al. 2015a, Balvín and Booth 2018, Holleman et al. 2019, Lewis et al. 2023). In addition, mutations were found in the III–IV interlinker of the VGSC of *C. lectularius* (F1524C) and *C. hemipterus* (F1450C). The change of this amino acid has been previously reported to confer moderate pyrethroid resistance in the yellow fever mosquito, *A. aegypti* (Kandel et al. 2019, Yougang et al. 2020), and the tiger mosquito, *A. albopictus* (Modak and Saha, 2022). The location of these mutations was also consistent across several insects, including cockroaches and lice, and their presence highlights the possible selective pressure

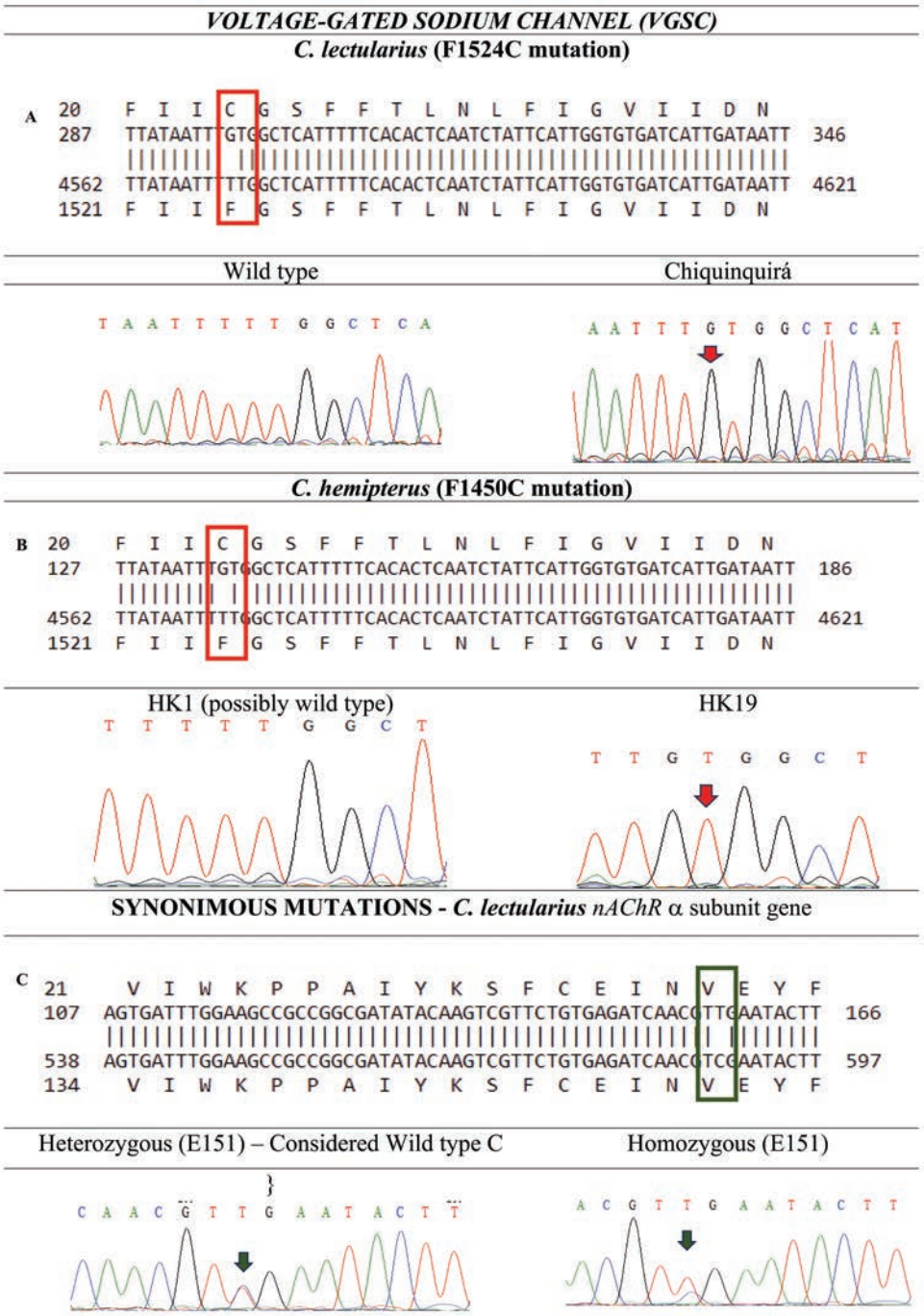


**Fig. 4.** Alignments and chromatograms of the amino acid sequence of *VGSC* and *nAChR* genes of *C. lectularius* and *C. hemipterus.* **A)** F1524C change in *C. lectularius*; the mutation is indicated by a box and an arrow. **B)** F1450C change in *C. hemipterus.* HK1 (no change) and HK19 were compared with each other. **C).** Synonymous mutations (E151) in the α subunit B-loop of the *nAChR* gene of *C. lectularius.* **D)** Synonymous mutations (G191 and V211) in the α subunit B-loop of the *nAChR* gene of *C. hemipterus.* These samples were compared with a *C. lectularius* (Fort-Dix) sequence found in GenBank with the accession number XM_014389050.2. **E)** Synonymous mutations (L59) of the β subunit D-loop of the *nAChR* gene of *C. hemipterus.*

exerted by the pyrethroid insecticides. Future research should focus on conducting functional assays in bed bugs to elucidate the exact role of these mutations in conferring resistance. The presence of the F1524C in *C. lectularius* samples that also contain other mutations warrants a new notation with a superscript +, that is, the sample B1.38 (L925I, F1524C) could be denoted $B^{+}$, whereas Chiquinquirá (I936F, F1524C), $A^{b+}$. These amino acid substitutions could be the result of selection upon existing standing variation, or alternatively mutation arising following the introduction of the insecticidal selection pressure. However, it is important to note that at present the importance of the mutation found in this interlinker (III-IV) is unknown. Finally, our study identified several synonymous mutations in the *VGSC* genes and *nAChR* of *C. hemipterus*, but the impact of these mutations for insects remains unclear.

Genetic analysis from *C. lectularius* populations collected in the European countries represents the most recent data on *kdr* mutations from this continent. In our analysis of samples from Spain, 18% were haplotype A, 4.7% were $A^{b}$, 74% haplotype B, and 8% haplotype C.

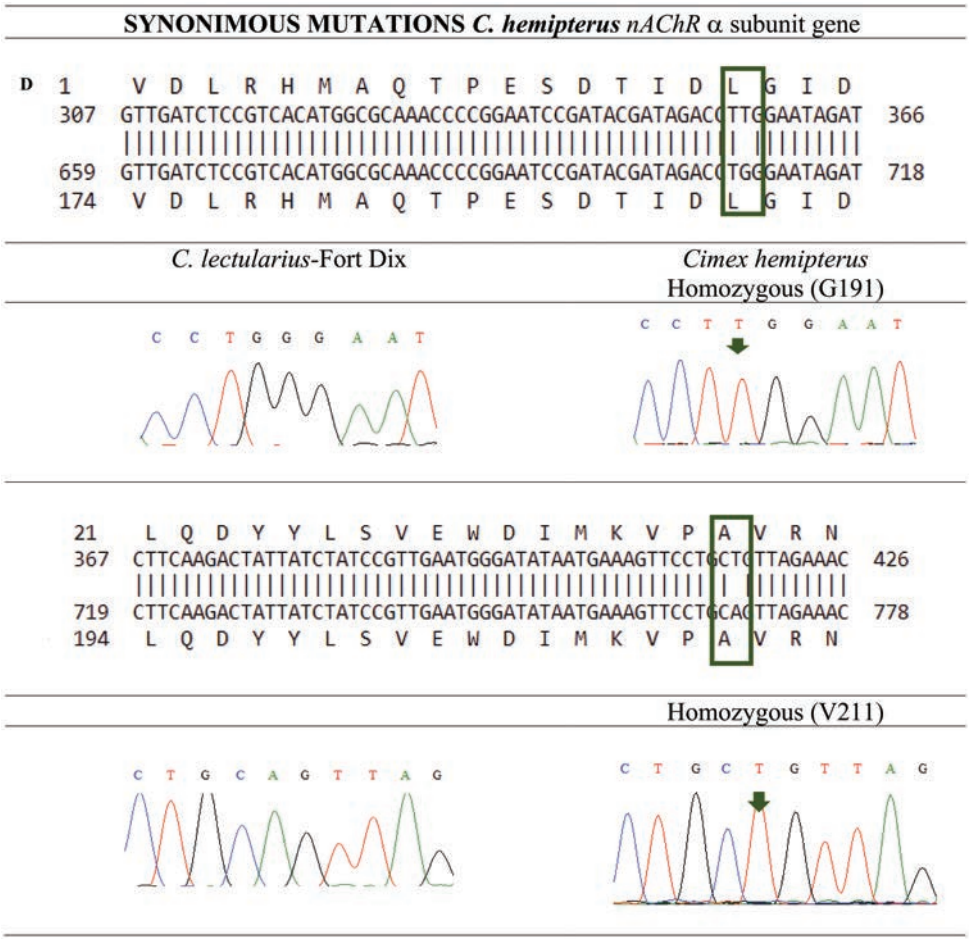


**Fig. 4.** Continued

*Cimex lectularius* from Switzerland (2 populations) and the Czech Republic (1 population) exhibited haplotype B. Comparison of all our European samples to those reported by Balvín and Booth (2018) for 14 European countries shows that while the frequency of haplotype A was similar, the frequency distribution of the rest of the haplotypes differed. For example, the frequency of haplotype B (containing only L925I) in our samples (73.3%) was lower than that reported by Balvín and Booth in 2018 (93.3%). Conversely, the frequency of haplotype C (containing both L925I and V419L) (8%) was higher than that reported by the same authors (1.9%). However, the minimal overlap of samples between the 2 studies (93.2% of our European samples came from Spain; 49.5% of the samples in Balvín and Booth 2018 came from the Czech Republic and none from Spain) might support the hypotheses related to geographical differences across European countries in the sources of bed bug populations or present-day selection pressures they experience.

Unlike *C. lectularius* from Europe, bed bugs analyzed from the United States came from laboratory colonies that were established from specimens collected from field populations during the period 2008–2022. Hence, the mutation and enzyme profiles of these colonies may not accurately reflect their resistance status at the time of collection from the field. From the 14 analyzed samples, 11 (~78%) were collected between 2017 and 2022; therefore, this analysis represents the most recent report of *kdr*-associated mutations from bed bug populations from the United States. Regardless, the collective analysis of haplotypes (2008–2020) indicated that haplotype C (L925I and V419L) was the most prevalent (85.7%), whereas only 1 population (7%) exhibited the L925I mutation (haplotype B). These results are congruent with a report about the predominance of haplotype C in bed bug populations in the United States in the last decade (Holleman et al. 2019, Lewis et al. 2023). Holleman et al. (2019). Temporal changes in the frequency of resistant haplotypes in the bed bug populations from the United States was initially reported by Lewis et al. (2023) when they compared the *kdr*-genetic profile of nearly 300 populations collected in 2 different time periods (2005–2009 and 2018–2019). This analysis showed that haplotype C increased by 68% within a decade, a situation that was attributed to intense selection of resistant populations by pyrethroid-based treatments for bed bug control (Lewis et al. 2023).

We screened 3 bed bug populations collected in poultry houses for *kdr* mutations, and all exhibited haplotype C. To our knowledge, this is the first report on the presence of *kdr* mutations in *C. lectularius* populations from the poultry farms. It is not clear, however, whether the presence of *kdr* mutations in bed bugs in the poultry farms is related to local on-farm selection with pyrethroids, or recurrent introductions of propagules by workers, supplies, feed, and other human-mediated routes. The detection of *kdr*-associated mutations in these samples suggests that treatments with pyrethroid-based formulations could be ineffective in these places. Indeed, only 4% of Winfield bed bugs (collected in a poultry farm in 2021) died after exposure to an $LD_{99}$ dose (11.8 ng) of deltamethrin (González-Morales et al. 2022). Where pyrethroid resistance is an issue, novel

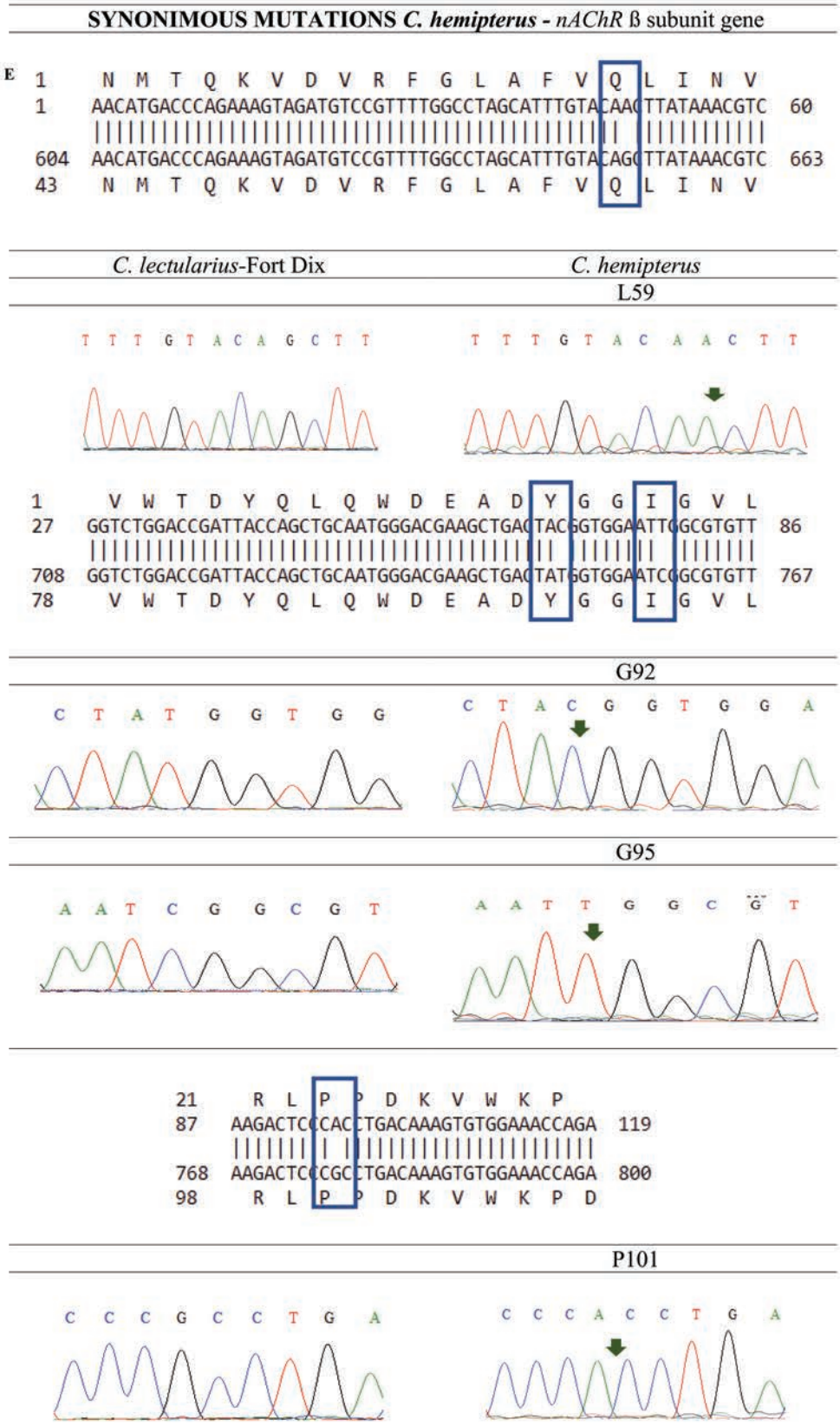


**Fig. 4.** Continued

strategies, such as the systemic antiparasitic drug fluralaner, have demonstrated a great potential for its practical use to control bed bug infestations in poultry farms (Matos et al. 2017, Sierras and Schal 2017, González-Morales et al. 2022).

Three Mexican *C. lectularius* samples were collected in the US-bordering city of Juarez, 2 of which exhibited haplotype B (L925I) and 1 haplotype C (L925I and V419L). Although the number of screened samples was low in Mexico, these results provide evidence

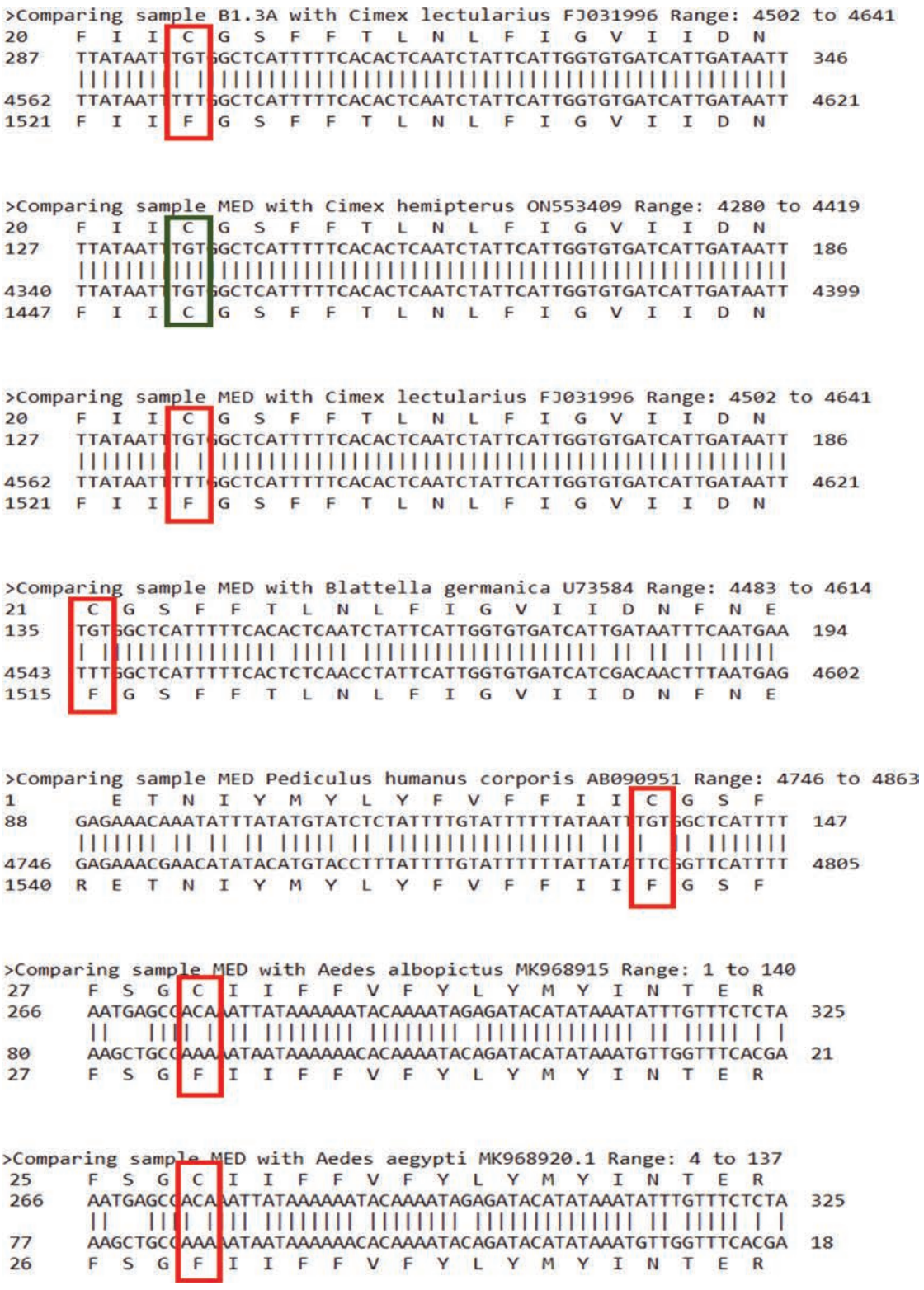


**Fig. 5.** Alignments of DNA and protein sequences from *VGSC* genes of *Cimex spp.* with NCBI reference *VGSC* genes. **A**) *Cimex lectularius* B1.38 (Madrid, Spain) with a wild type of the same species; **B**) *C. hemipterus* MED (Medellin, Colombia) with the mRNA sequence of *C. hemipterus* from GenBank to identify the aminoacid position (C1450); **C**) MED with a wild-type *C. lectularius*; **D**) *B. germanica*; **E**) *P. humanus corporis*; **F**) *A. albopictus*; and **G**) *A. aegypti.*

that populations with *kdr*-based insecticide resistance are already present in Mexico. The close proximity to the US-Mexican border, an area with intensive commercial trade and cross-border human connectivity and mobility, makes it premature to infer evolution of insecticide resistance in bed bug populations from Mexico

We also analyzed the enzymatic profiles of the resistance-associated enzymes in the collections made in the United States and Mexico. Higher activity of P450 (relative to the reference susceptible Ft. Dix bed bugs) was detected in Cincinnati, a population with no *kdr* mutations, and Shanda, a population from North Carolina

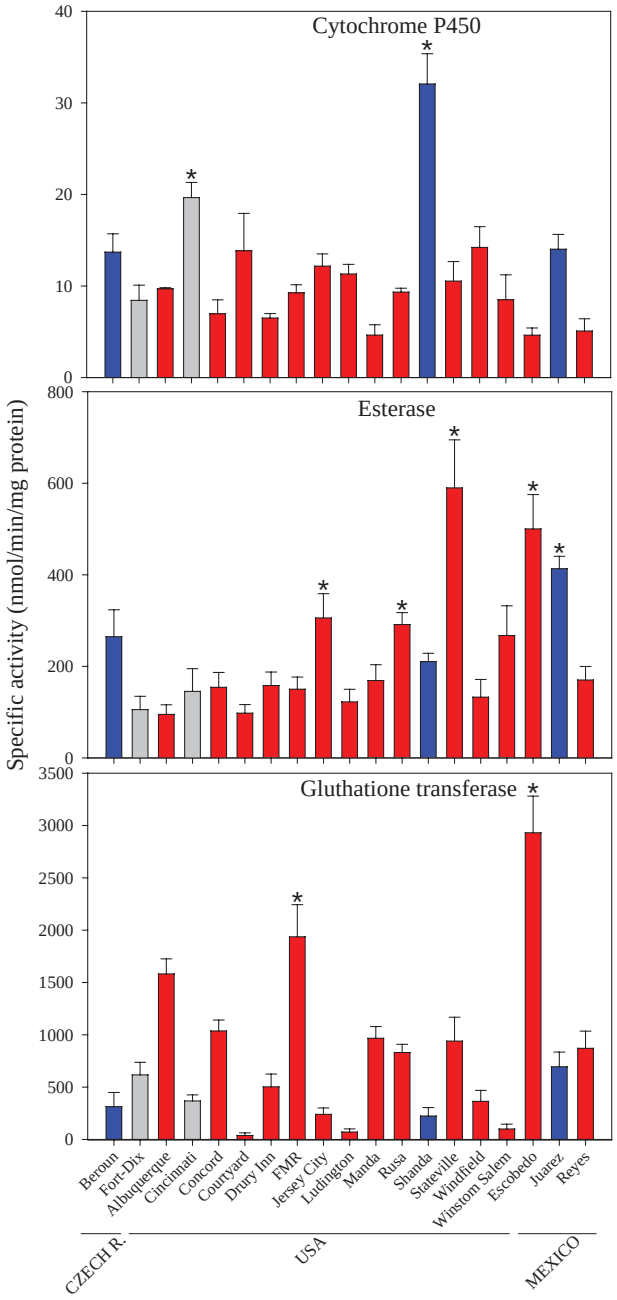


**Fig. 6.** Comparison of cytochrome P450 monooxygenase (P450), general esterase (EST), and GST activities of insecticide-susceptible bed bugs (Fort-Dix Strain) and 19 bed bug populations. Bars indicate the activity change in enzyme activity of bed bug strains relative to the susceptible strain (Fort-Dix).

that contained L925I (haplotype B). The activity of esterases was increased in 4 out of 14 populations exhibiting haplotype C, whereas the activity of GSTs was increased in 2 out of 14 populations. These data suggest that some common bed bug populations have developed multiple insecticide-resistance mechanisms that could reduce the efficacy of insecticide treatments (Adelman et al. 2011, Zhu et al. 2013, Romero and Anderson 2016).

Only 3 Colombian samples of *C. lectularius* were screened for resistance, and all contained *kdr*-resistance-associated mutations. One sample collected in Bogotá, exhibited both L925I and V419L (haplotype C), 1 collected in Tunja L925I and I936F (haplotype $B^b$), and 1 collected in Chiquinquira I936F ($A^b$). *Cimex lectularius* populations that exhibit I936F have been detected at low percentages in countries such as Australia (Dang et al. 2015a), Israel (Palenchar et al. 2015), the United States (Holleman et al. 2019), and Spain (this study). Discovery of a bed bug population containing L925I and I936F (haplotype $B^b$) in Colombia could represent the genetic diversity present in the founding propagules, which might have originated from countries where similar genetic resistance profile has been reported. In Australia, samples with haplotype $B^b$ were also reported, but there is no information about their correlation with pyrethroid resistance (Dang et al. 2015a). However, it should be expected that populations with these *kdr* genotypes are resistant to pyrethroids due to the presence of the L925I substitution, which itself confers high levels of pyrethroid resistance (Zhu et al. 2010).

All 34 samples of *C. hemipterus* collected in Hong Kong possessed 3 of the 12 previously reported amino acid substitutions; namely M918I, D953G, and L1014F. The M918I mutation is strongly associated with *kdr*- or super-*kdr*-type resistance to pyrethroids in many insect pests (Chen et al. 2017). The L1014F mutation, originally found in *Musca domestica* L. (Diptera: Muscidae), also confers high levels of pyrethroid resistance (Smith et al. 1997). These 2 mutations have been detected in *C. hemipterus* collections from Australia, India (Dang et al. 2015b), China (Zhao et al. 2020), Iran (Ghavami et al. 2021), and Korea (Cho et al. 2023), among other countries, and have been correlated with moderate-to-high levels of resistance to pyrethroids. We also detected the D953G mutation, which has been reported in conjunction with M918I and L1014F mutations in *C. hemipterus* populations from Thailand and Korea, but its contribution to pyrethroid resistance has not been confirmed (Dang et al. 2015b, 2021, Cho et al. 2023). Interestingly, whereas we found 3 mutations (M918I, D953G, and L1014F) in all of our 34 *C. hemipterus* samples collected in 2022 from Hong Kong, Zhao et al. (2020) reported only 2 of such mutations (M918I and L1014F) in 6 *C. hemipterus* populations collected in the Guangxi Zhuang Region of China. However, Zhao et al. (2020) did not amplify the fragment of the *VGSC* gene where the D953G mutation has been previously reported.

In our study, we screened the B and D loops of the *nAChR* gene, areas where neonicotinoid mutations have been reported for other insects (Liu et al. 2005, Hirata et al. 2015). Our screen of the *nAChR* gene did not find any mutations in all tested populations, including Cincinnati, a population that exhibited high levels of resistance to various neonicotinoids (Romero and Anderson 2016) and showed elevated P450 activity in the current study. Future studies should expand the search of mutations to other regions of the *nAChR* gene, including the transmembrane domain 3, which confers resistance to spynosins through the G275E mutation (Puinean et al. 2013).

While bed bug infestations continue to rise worldwide, there is a noticeable lack of intent among governments in many countries to implement surveillance and education programs that would lay the foundation for effective bed bug management and prevention strategies. This hesitancy is primarily rooted in the perception that bed bugs are merely a nuisance, as there is currently no evidence supporting their transmission of disease-causing pathogens. Compounding the issue is the public's widespread unawareness of bed bugs, contributing inevitably to their proliferation in various regions globally. These challenges are further exacerbated by the presence of insecticide resistance, creating fertile grounds for the escalation of the problem. More contemporary data on bed bugs in Mexico, Colombia, and other Latin American countries will hopefully encourage the establishment of effective public policies for the prevention, monitoring, and control of bed bugs.

## Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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Julián Porras (Conceptualization [equal], Data curation [equal], Formal analysis [equal], Investigation [equal], Methodology [equal], Project administration [equal], Writing—original draft [equal], Writing—review & editing [equal]), Immo Hansen (Methodology [equal], Project administration [equal], Resources [equal], Supervision [equal], Writing—review & editing [equal]), Lee Uranga (Methodology [equal], Resources [equal], Writing—review & editing [equal]), Matthew Pinch (Conceptualization [equal], Methodology [equal], Supervision [equal], Writing—review & editing [equal]), Coby Schal (Resources [equal], Writing—review & editing [equal]), Sandra Sáez-Durán (Conceptualization [equal], Project administration [equal], Resources [equal], Writing—review & editing [equal]), Dr. Ruben Bueno (Conceptualization [equal], Project administration [equal], Resources [equal], Supervision [equal], Writing—review & editing [equal]), MARIA TRELIS (Conceptualization [equal], Methodology [equal], Project administration [equal], Resources [equal], Writing—review & editing [equal]), Màrius V. Fuentes (Conceptualization [equal], Methodology [equal], Project administration [equal], Resources [equal], Supervision [equal], Writing—original draft [equal], Writing—review & editing [equal]), Sudip Gaire (Methodology [equal], Writing—review & editing [equal]), and Alvaro Romero (Conceptualization [equal], Data curation [equal], Methodology [equal], Project administration [equal], Resources [equal], Writing—original draft [equal], Writing—review & editing [EQUAL])

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